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Short communication

Dimerization and protease resistance: New insight into the function of PR-1☆

Shunwen Lu[a,*], Justin D. Faris[a], Robert Sherwood[b], Michael C. Edwards[a]

[a] USDA-ARS, Cereal Crops Research Unit, Fargo, ND 58102, USA
[b] Cornell University Life Sciences Core Laboratories Center, Ithaca, NY 14853, USA

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ABSTRACT

The group 1 pathogenesis-related (PR-1) proteins have long been considered hallmarks of hypersensitive response/defense pathways in plants, but their biochemical functions are still obscure despite resolution of the NMR/X-ray structures of several PR-1-like proteins, including P14a (the prototype PR-1). We report here the characterization of two basic PR-1 proteins (PR-1-1 and PR-1-5) recently identified from hexaploid wheat (*Triticum aestivum*). Both proteins were expressed in *Pichia pastoris* as a single major species of ~15 kDa. Sequence identity of the expressed PR-1 proteins was verified by MALDI-TOF/TOF analysis. Accumulation of the native PR-1-5 protein in pathogen-challenged wheat was confirmed by protein gel blot analysis. Low-temperature SDS-PAGE and yeast two-hybrid assays revealed that PR-1-1 exists primarily as a monomer whereas PR-1-5 forms homodimers. Both PR-1 proteins are resistant to proteases compared to bovine serum albumin, but PR-1-1 shows resistance mainly to subtilisin and protease K (serine proteases) whereas PR-1-5 shows resistance to subtilisin, protease K and papain (a cysteine protease). Site-specific mutations at the five putative active sites in the PR-1 domain all affected dimerization, with the mutations at Glu-72 and Glu-102 (in the PR-1-5 numeration) also diminishing protease resistance. Sequence analysis revealed that the Glu-72 and Glu-102 residues are located in motif-like sequences that are conserved in both PR-1 and the human apoptosis-related caspase proteins. These findings prompt us to examine the function of PR-1 for a role in protease-mediated programmed cell death pathways in plants.

Introduction

Pathogenesis-related (PR) proteins consist of at least 17 families of proteins that are induced in plants upon infection by microbial pathogens or attack by insects. The PR-1 family was first identified as early as 1970 from *Tobacco mosaic virus*-infected tobacco plants showing HR symptoms (van Loon and van Kammen, 1970), and has been since considered a hallmark of plant defense and HR-type PCD pathways (Buchel and Linthorst, 1999; van Loon and van Strien, 1999; van Loon et al., 2006). Recent studies have shown that plant genomes contain multigene families encoding PR-1-like proteins (van Loon et al., 2006; Mitsuhara et al., 2008; Lu et al., 2011). Homologues of PR-1 proteins are also found in other eukaryotic organisms, including fungi, insects, animals, and humans (Gibbs et al., 2008) with the best known example being the glioma pathogenesis-related (GliPR) protein implicated in cancer development in human cells (Szyperski et al., 1998).

Plant PR-1 proteins are classified as acidic or basic based on their isoelectric points (van Loon and van Strien, 1999). Both groups of PR-1 proteins have been purified from various plant species including tobacco and tomato (Bol and Linthorst, 1990) and barley (Bryngelsson et al., 1994). Some basic PR-1 proteins possess antifungal activities, *e.g.*, the tomato P14c and tobacco PR-1g proteins (Niderman et al., 1995). The fact that PR-1 proteins are pathogen-inducible and frequently isolated from HR-associated plant tissues supports a role for PR-1 in PCD-related pathways, but the underlying mechanisms are still unknown. To date, the biochemical functions of PR-1-like proteins have not been resolved.

All PR-1-like proteins contain a conserved PR-1 domain (also called the SCP-like extracellular protein domain, pfam00188) featuring four α-helices and one four-strand β-sheet as represented by the tomato PR-1 protein P14a (Fernandez et al., 1997) and the human GliPR (Szyperski et al., 1998) proteins in which His-72, Glu-77, Glu-98 and His-117 (in the P14a numeration) are identified to be potential active sites. Homology-based modeling for the cone snail

Abbreviations: BSA, bovine serum albumin; HR, hypersensitive response; *HRP*, horseradish peroxidase; Lt-SDS-PAGE, low temperature-SDS-PAGE; PCD, programmed cell death; PCK1, phosphoenolpyruvate carboxykinase; SP, signal peptide; Y2H, yeast two-hybrid; WT, wild type.

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* Corresponding author at: USDA-ARS, Cereal Crops Research Unit, Northern Crop Science Laboratory, Fargo, ND 58102-2765, USA. Tel.: +1 701 239 1344; fax: +1 701 239 1369.
E-mail address: Shunwen.Lu@ars.usda.gov (S. Lu).

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PR-1-like protein Tex31 identified Ser-73, Glu-98 and His-117 as a "catalytic triad" reminiscent of that of serine proteases (Milne et al., 2003). A different "catalytic triad" was proposed for the human Golgi-associated pathogenesis-related (GAPR-1) protein in which Ser-73 is thought to contact His-72 and Glu-77 upon formation of a dimerization interface (Serrano et al., 2004). These brought up a total of five putative active sites in the PR-1 domain that suggest an enzymatic function. However, the importance of individual active sites with respect to function has not been demonstrated in PR-1-like proteins.

The genome of hexaploid wheat (*Triticum aestivum*) contains at least 23 *PR-1*-like genes that encode three major groups of PR-1-like proteins (basic, basic with a C-terminal extension, and acidic), with a majority induced or up-regulated upon pathogen attacks (Lu et al., 2011). To help unravel the role of PR-1 in host-pathogen interactions, we explored the biochemical functions of the wheat PR-1 proteins using *in vitro* approaches. Here we report the heterologous expression and functional characterization of PR-1-1 and PR-1-5 from the basic PR-1 group. We provide evidence that the two basic PR-1 proteins exist as both monomers and dimers; both forms are resistant to proteolytic attack, and such resistance relates to dimerization which depends on the putative active sites in the PR-1 domain that harbors motif-like sequences conserved in apoptosis-related caspase-like cysteine proteases.

Materials and methods

Expression of PR-1 proteins in yeast

The coding regions (minus SP) of *PR-1-1* (GenBank accession HQ541961) and *PR-1-5* (HQ541965) were PCR-amplified using primers MlyI-pr1F 5′-CGCGAGTCTCATGCAGAACTCGCCTCAGGACT-3′ with KpnI-pr1R 5′-CGGGGTACCTTAGTATGGTTTCTGTCCAACAAC-3′, and MlyI-pr5F 5′-CGCGAGTCTCATGCAGAATACGCCGCAGGACT-3′ with KpnI-pr5R 5′-CGGGGTACCCTAGTATGGTTTCTGTCCAATG-3′, respectively, and subcloned into the pPinkα-HC vector (Invitrogen). Site-specific mutations were generated in PR-1-5 by PCR fusion with primers MlyI-pr5F and KpnI-pr5R, each in combination with a primer containing the targeted mutation site. Constructs were transformed into an Invitrogen strain of *Pichia pastoris* (Guillierm.) Phaff. Secreted proteins were desalted through a Bio-Gel P-6DG column (Bio-Rad), separated by SDS-PAGE on 15% polyacrylamide gels and visualized by *Coomassie Blue staining*. Concentration was estimated by comparison with a series of dilutions of BSA. Protein bands were excised from gels and sequence identity was confirmed by MALDI-TOF/TOF analysis at Cornell University Life Sciences Core Laboratories Center.

Detection of PR-1 protein dimers

Lt-SDS-PAGE was performed as described (Lu et al., 2008) with some modifications: protein samples were incubated at room temperature for 10 min, and then kept on ice for 30 min before loading in 2 × SDS sample buffer lacking reducing agent for preservation of dimerization, or in 2 × SDS sample buffer containing β-mercaptoethanol heated at 95 °C for 5 min for denaturation. Y2H assays were performed using the GAL4-based system (Clontech) with the pGADT7 (prey) or the pGBKT7 (bait) vectors as described (Lu, 2012).

Plant protein extraction and protein gel blot analysis

Total proteins were extracted from homogenized leaf tissues (~100 mg) of two-week-old wheat (*Triticum aestivum* L.) cv Grandin that were either healthy or infected by the leaf/glume blotch fungal pathogen *Phaeosphaeria nodorum* (E. Müll.) Hedjar as described previously (Lu et al., 2011) with 1 mL of buffer solution containing 25 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol and 2% polyvinylpolypyrrolidone. *HRP*-conjugated PR-1-5-specific polyclonal antibody was produced by GenScript (www.genscript.com) using a synthetic peptide (AWVGEKQDYDYGSNTC) (attempt to develop an anti-PR-1-1 antibody was unsuccessful). Antibody against the maize PCK1 was purchased from Novus Biologicals. Signals were detected with Western Chemiluminescent HRP Substrates (Millipore) followed by exposure to Kodak X-ray films.

Protease resistance assays

Subtilisin, protease K and papain were purchased from Promega, NEB and Sigma, respectively. Digestions were performed in a volume of 20–40 μL containing 0.2–0.5 μg of targeted protein (or ~20.0 μg of wheat total protein) and a protease in a series of dilutions with a final concentration 2×10^{-4} to 0.05 μg μL^{-1}. A higher concentration (0.5 μg μL^{-1}) was also used for subtilisin digestion of plant proteins (Fig. 3B). After incubation at room temperature overnight, the protease was inactivated by heating at 95 °C for 5 min. BSA (66.4 kDa) and the protease inhibitor aprotinin (Sigma, 6.5 kDa) were used as protease-sensitive, and -resistant controls, respectively.

Results

Characterization of recombinant PR-1 proteins

Both PR-1-1 and PR-1-5 proteins were expressed (separately) in yeast as a single major species with an expected molecular mass of ~15 kDa; no significant background secretions were detected from a "mock" yeast strain transformed with the expression vector only (Fig. 1A, top). The amount of each recombinant PR-1 protein was estimated at ~0.01 μg μL^{-1}. The anti-PR-1-5 antibody gave a signal only when hybridized to the 15-kDa PR-1-5 protein (Fig. 1A, bottom, lane 2), and detected a 15-kDa band only from the protein sample isolated from the infected plants (Fig. 1B, bottom, lane 2). MALDI-TOF/TOF analysis identified at least seven of the 10 predicted tryptic fragments that cover >80 residues with perfect matches to the deduced PR-1-1/PR-1-5 protein sequences including the first 15 residues at the N-terminus of the recombinant protein (Fig. 1C).

PR-1-5 protein forms homodimers

PR-1-1 was found to be mainly a monomer as indicated by the presence of the 15-kDa band characteristic of a monomeric protein along with a faint band at >25 kDa predicted to represent a dimeric protein (Fig. 2A, lane 1) whereas PR-1-5 formed predominantly dimers as indicated by the presence of a major band at >25 kDa along with a faint 15-kDa band under non-denaturing conditions (lane 2); both proteins gave a single 15-kDa band under denaturing conditions (lanes 3 and 4). MALDI-TOF/TOF analysis confirmed that the >25 kDa band consisted of PR-1-5 peptides only; no other unrelated peptides were detected. Y2H assays indicated that a positive interaction only occurred between the two "monomeric" PR-1-5 proteins (each used as the "bait" or the "prey"); no interactions were detected between a PR-1-1 "bait" and a PR-1-5 "prey" protein (Fig. 2B). Since PR-1-1 is highly similar (94% similarity) to PR-1-5, but failed to substitute the later for the activation of the reporter genes, the result suggested strongly that PR-1-5 forms "homodimers" *in vivo*, at least in yeast.

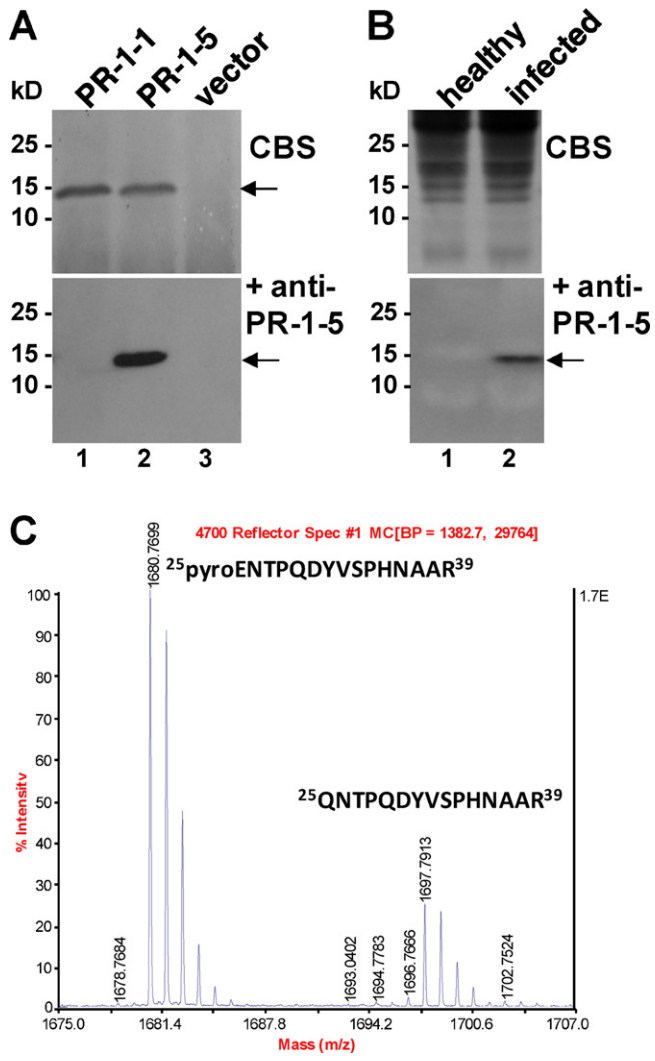

Fig. 1. Characterization of PR-1-1 and PR-1-5 proteins. (A) Coomassie Blue-stained SDS-PAGE gel (CBS, top) showing the ~15 kDa PR-1-1 and PR-1-5 proteins (arrow) expressed in yeast, and a protein gel blot (bottom) showing the antibody specificity to PR-1-5 (arrow). (B) SDS-PAGE gel (top) showing total wheat proteins, and a protein gel blot (bottom) showing the accumulation of PR-1-5 (arrow) in pathogen challenged wheat. (C) MS/MS spectra showing the N-terminal peptides of the PR-1-5 protein that include the "native" form with the N-terminal glutatmine (Q) (peak at 1696.78 Da M+H) and the "modified" form, in which the Q residue underwent a spontaneous cyclization to pyroglutamic acid (pyroE) (peak at 1680.77 Da M+H). Numbers at the ends of the sequences indicate the positions in the full-length PR-1-5 protein. The presence of both types of peptides in the PR-1-1 protein sample was also confirmed (not shown). Numbers on the left in (A) and (B) indicate the molecular masses (in kDa) of protein markers.

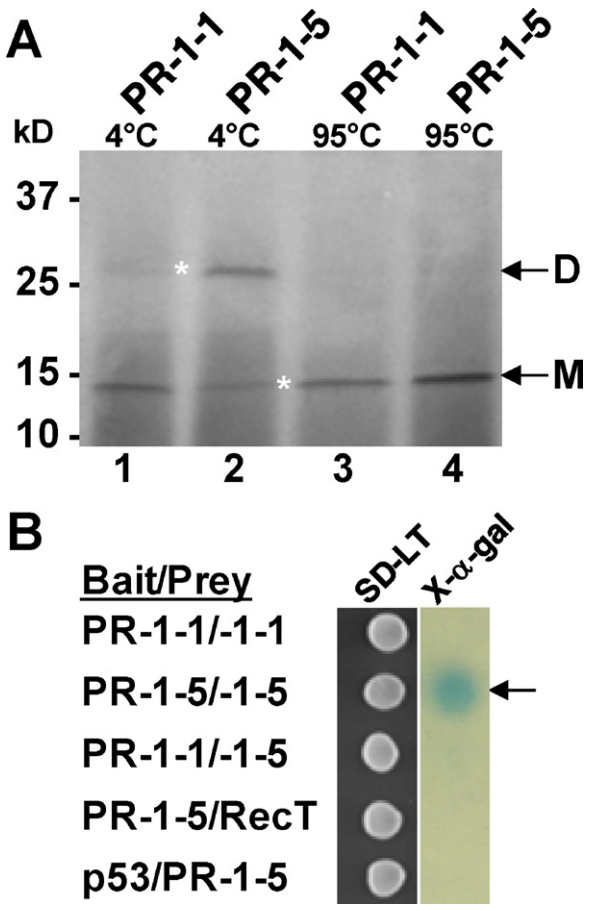

Fig. 2. Dimerization of the PR-1-1 and PR-1-5 proteins. (A) Coomassie Blue-stained Lt-SDS-PAGE gel showing the co-existence of PR-1-1 and PR-1-5 monomers (M, ~15 kDa) and dimers (D, >25 kDa) under non-denaturing conditions (4 °C), and the presence of the corresponding monomers under denaturing conditions (95 °C). Weak bands (asterisks) indicate a much lower amount of PR-1-1 dimers (lane 1) and PR-1-5 monomers (lane 2), respectively. Numbers on the left indicate the molecular masses (in kDa) of protein markers. (B) Y2H assays. Transformants co-expressing the bait and the prey constructs were inoculated onto synthetic defined medium (SD) without leucine and tryptophan (-LT) which selects for the bait and prey proteins, or supplemented with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal) without adenine and histidine for the yeast reporter gene encoding the α-galactosidase. Blue coloration (arrow) indicates a "positive" interaction in the transformant co-expressing the "monomeric" bait/prey PR-1-5 proteins.

PR-1 proteins are resistant to proteolytic attack

Both PR-1-1 and PR-1-5 proteins showed resistance to subtilisin within the final concentration range of 2×10^{-4} to 0.013 $\mu g\,\mu L^{-1}$ (Fig. 3A, top). Resistance to protease K was also observed for the two PR-1 proteins (data not shown). PR-1-5 also showed resistance to papain until the concentration reached 6×10^{-3} $\mu g\,\mu L^{-1}$ whereas PR-1-1 appeared to be sensitive to the same enzyme with digestion clearly occurring at 8×10^{-4} $\mu g\,\mu L^{-1}$, 16-fold lower than that for digestion of PR-1-5 (Fig. 3A, bottom). The native PR-1-5 protein was also resistant to subtilisin: the PR-1-5-specific 15-kDa band detected in the pathogen-challenged plants showed gradually decreased signal intensity, but remained visible until the concentration reached 0.025 $\mu g\,\mu L^{-1}$ (Fig. 3B, top, lanes 2–7) whereas the "house-keeping" PCK1 protein (found in both healthy and pathogen-challenged plants) was completely digested at 1.6×10^{-3} $\mu g\,\mu L^{-1}$ (Fig. 3B, middle), >30-fold lower than the concentration needed for digestion of the native PR-1-5 protein. The total wheat proteins appeared to be fully digested at enzyme concentrations > 1.6×10^{-3} $\mu g\,\mu L^{-1}$ as shown on the Coomassie Blue-stained PVDF membrane (Fig. 3B, bottom). No reduced proteolytic activity was observed when PR-1-1 or PR-1-5 was pre-incubated with subtilisin (or protease K) before digesting other proteins (*e.g.*, BSA); neither PR-1 proteins (or the same amount of "secretions" from the "mock" strain) gave positive results when tested for ability to degrade common substrates (*e.g.*, casein or gelatin) (data not shown).

Active sites in the PR-1 domain mediate dimerization and protease resistance

Five PR-1-5 mutant proteins, each carrying a single alanine replacement at His-72, Ser-73, Glu-79, Glu-102 and His-121, respectively, were expressed in yeast. All five mutations affected dimerization of the PR-1-5 protein (Fig. 4A). The H72A mutation

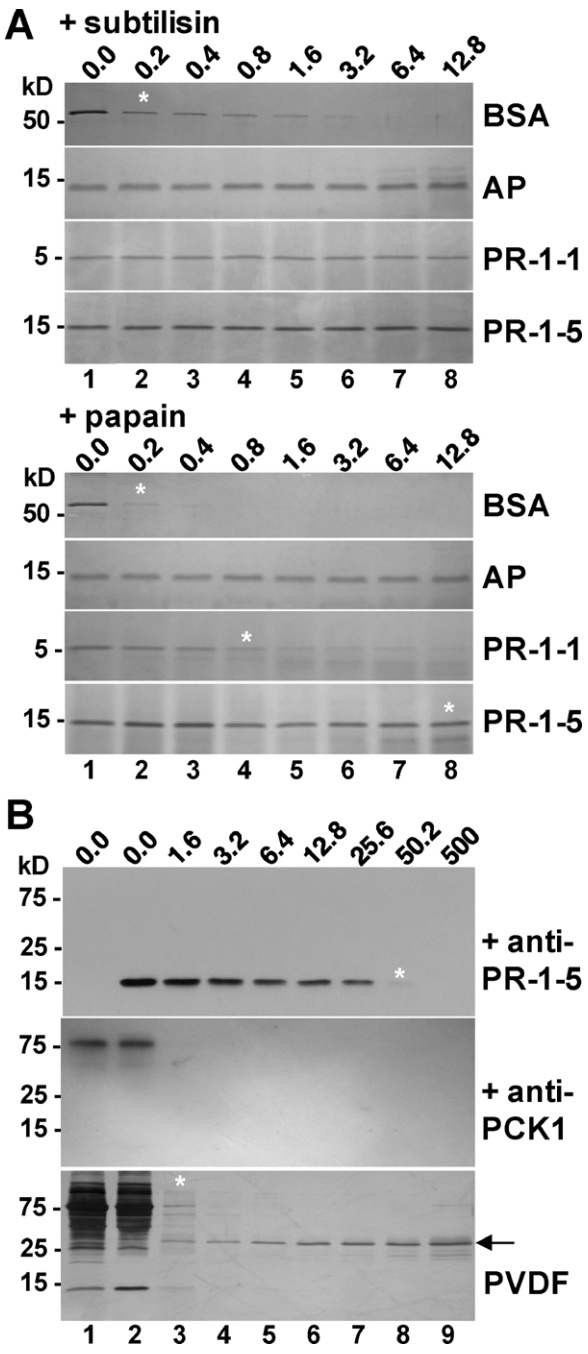

Fig. 3. PR-1-1 and PR-1-5 proteins are resistant to proteolytic attack. (A) Coomassie Blue-stained SDS-PAGE gels showing digestions with subtilisin and papain of ~0.5 μg of PR-1-1, PR-1-5 and two control proteins BSA and aprotinin (AP). (B) Protein gel blots showing subtilisin digestion of total proteins (~20 μg per lane) of healthy (lane 1) or pathogen-challenged (lanes 2–9) wheat plants. The same blot was probed first with anti-PR-1-5 antibody (top panel), and then with anti-PCK1 antibody (second panel) after stripping, finally stained with Commassie Blue (bottom panel). Arrow indicates subtilisin (~35 kDa). Numbers at the top indicate the final concentrations of proteases (ng μL^{-1}). Asterisk indicates a point where an apparent digestion occurred. Numbers on the left indicate the molecular masses (in kDa) of protein markers.

(lane 2) resulted in an apparent molecular mass slightly higher than that of the WT PR-1-5 dimer (lane 1) whereas the proteins derived from the S73A (lane 3) and E79A (lane 4) mutations migrated as two forms: one appeared to be a WT dimer and the other migrated to a position between the WT monomer and dimer. These aberrant bands were likely due to mutation-related conformational changes since proteins from all these mutants migrated as 15-kDa monomeric bands under denaturing conditions (Fig. 4B, top panel). The E102A mutant protein (lane 5) migrated to a position corresponding to the WT monomer (15 kDa), but with much less dimer formation under non-denaturing conditions. The H121A mutant protein (lane 6) migrated to a position >37 kDa, likely indicating the formation of a trimer. When tested for protease resistance (Fig. 4B), the H72A and H121A mutants remained resistant to subtilisin, protease K, and papain like the WT PR-1-5 protein, but the other three mutants suffered a reduction in resistance to all three proteases with varying degrees. The S73A mutant protein band showed slightly reduced staining intensity in all three digestions. The E79A mutant showed a reduction in resistance to subtilisin that was not obvious due to the relatively lower concentration of the mutant protein, but appeared to be completely digested by protease K and papain. The E102A mutant sustained greatly reduced resistance to subtilisin, and was completely digested by protease K and papain.

PR-1 and human caspase proteins share conserved motif-like sequences

We explored the relatedness between the active sites and function in the PR-1 domain by searching for PR-1-like "active sites" in subtilisin-like serine proteases and caspase-like cysteine proteases that are built on an α–β–α sandwich structure (Madala et al., 2010). Four motif-like sequences were found to be conserved between PR-1 and human caspase 3 (CASP3) and caspase 7 (CASP7) in the cysteine protease group that play key roles in human/animal PCD pathways (Mittl et al., 1997; Chai et al., 2001). These include "EN#X_2G" (Motif-1, # is an aliphatic residue), "(F/W)X_2(E/D)(K/R)" (Motif-2), "ϕX_3Y" (Motif-3, ϕ is a hydrophobic residue), and "GXϕ" (Motif-4) (Fig. 4C). Motifs 1 and 2 (which include Glu-79 and Glu-102, respectively) appeared to be most conserved: up to six continuous identical/similar residues were found in Motif-1 of the monocot PR-1 (ENIFWG) and CASP7 (ENVIYG) proteins, and the sequences around the catalytic residues H122 and C163 (in the CASP3 numeration) also showed conservation between plant PR-1 and CASP3/7 proteins (Fig. 4C).

Discussion

Among the 17 families of PR proteins recognized so far (van Loon et al., 2006), the PR-1 group is the only one that has not been assigned a biochemical function (*e.g.*, β-1,3-glucanase for PR-2 and chitinase for PR-3) or to a protein category with recognized functions. One major obstacle to elucidating the function of PR-1 is the genomic redundancy. There are estimated >60 *PR-1*-like genes in hexaploid wheat genome (Lu et al., 2011). Purification of individual native wheat PR-1 proteins from a pool of isoforms to homogeneity is a challenging task. In this study, we succeeded in expressing two PR-1 proteins using a yeast expression system, and obtained high yields and relative purity for both proteins (Fig. 1). This expression system will facilitate future studies of the wheat PR-1 protein family.

A recent study showed that the human GAPR-1 protein forms dimers (Serrano et al., 2004) but this PR-1-like protein is unusual in that it lacks the N-terminal SP and is associated with membrane (Groves et al., 2004). We demonstrated here that a secreted/extracellularly located plant PR-1 protein like PR-1-5 is capable of forming stable dimers both *in vitro* (in solution) and *in vivo* (at least in yeast cells), adding that both plant and animal PR-1-like proteins may regulate their functions through dimerization which is a common mechanism for activation and/or regulation of enzyme proteins including proteases (Marianayagam et al., 2004).

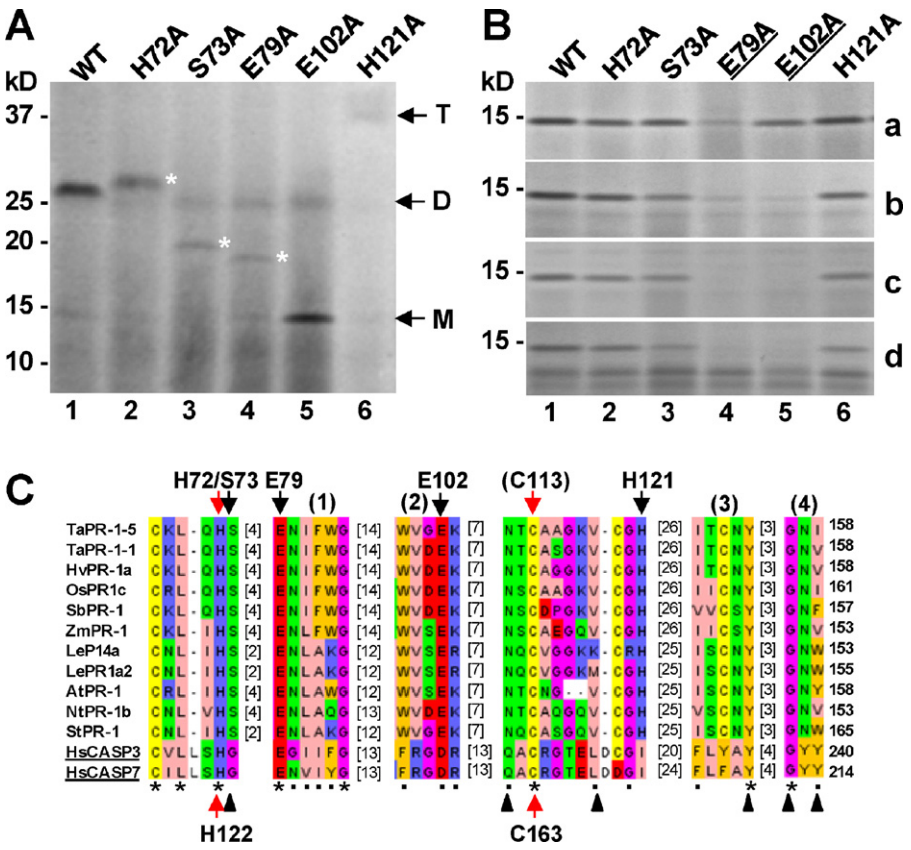

Fig. 4. Mutations at the five putative active sites in PR-1-5 affected dimerization and protease resistance. (A) Coomassie blue-stained Lt-SDS-PAGE gel showing the reduced dimerization of the mutant PR-1-5 proteins (lanes 2–6). Arrows indicate positions of monomer (M), dimer (D) and trimer (T). Unexpected bands are indicated by asterisks. (B) SDS-PAGE gels showing that the E79A and E102A mutations (underlined) greatly reduced or abolished resistance to three proteases. Each digestion contained ~0.5 μg of PR-1 protein (except for the E79A mutant, ~0.1 μg) and subtilisin (b), protease K (c) or papain (d) at a final concentration of 0.05 μg/μl. Panel a shows undigested controls. (C) Alignment of plant PR-1 proteins with human CASP3 and 7 (underlined, GenBank accession P42574 and P55210, respectively) proteins. Identical residues, conserved residues and active sites are indicated by asterisks, dots and arrows, respectively. Triangles indicate the substrate-binding sites in CASP3/7. Numbers with parentheses indicate motif-like sequences with distance numbered in brackets between sequences. It is to be determined if C113 (top, in parentheses) of PR-1 is an active site like C163 (bottom) in CASP3/7. Alignments were generated using MegAlign (DNASTAR). At, *Arabidopsis thaliana* (NM_127025); Hv, *Hordeum vulgare* (X74939); Le, *Lycopersicon esculentum* (M69248); Nt, *Nicotiana tabacum* (X05453); Os, *Oryza sativa* (EF061248); Sb, *Sorghum bicolor* (BE356947); St, *Solanum tuberosum* (AJ250136); Ta, *Triticum aestivum*; Zm, *Zea mays* (ZMU82200).

Protease resistance has not been demonstrated for any members of the PR-1 family. We showed that the recombinant PR-1-1 and PR-1-5 proteins are resistant to subtilisin and protease K, and the PR-1-5 also showed resistance to papain. We also confirmed that the native PR-1-5 protein is indeed protease-resistant, at least for subtilisin (Fig. 3). The fact that the dimeric PR-1-5 is more resistant to proteolytic attack than the monomeric PR-1-1 provides evidence that the dimerization regulates the function of PR-1. Several types of proteins are commonly known to be resistant to protease digestion, mainly proteases and protease inhibitors. For example, subtilisin and protease K are resistant to each other, although both are sensitive to papain digestion (data not shown). The pancreatic trypsin inhibitor aprotinin is highly resistant to all proteases we tested. Since both PR-1-1 and PR-1-5 show resistance to subtilisin and protease K with high degrees comparable with that of aprotinin (Fig. 3) but provided no protection to other proteins in this study, they are less likely to be protease inhibitors and more likely to be proteases.

Milne et al. (2003) demonstrated that the cone snail PR-1-like protein Tex31 (which is also protease-resistant) functions as a substrate-specific serine protease. But a subsequent study on the hookworm nematode Na-ASP-2 protein did not detect any serine protease-like activities (Asojo et al., 2005). In this study, we generated five PR-1-5 mutant proteins (each carrying a single amino acid replacement at one putative active site) and showed that the replacement of the proposed catalytic serine residue (Ser-73) with an alanine only caused slightly reduced resistance to the three proteases (Fig. 4), arguing that Ser-73 may not be the central catalytic residue for the "serine protease"-related activity. In contrast, mutations at Glu-72 and Glu-102 affected dimerization and resulted in severe loss of protease resistance (Fig. 4), suggesting that these two residues may play key roles in PR-1 function.

The identification of motif-like sequences common in PR-1 and human caspase (CASP) proteins (Fig. 4) possibly provided further clues for understanding the function of PR-1. CASPs are substrate-specific apoptosis-related cysteine proteases that include the "initiators" (*e.g.*, CASP8 and 9) and "executioners" (*e.g.*, CASP3 and 7) (Boatright and Salvesen, 2003). Mutations in Motif-1 (E79A) and Motif-2 (E102A) that are most conserved in PR-1 and CASP3/7 proteins resulted in the most severe impacts on the PR-1-5 protein, implying that plant PR-1 proteins may share certain functional commonality with CASP-like proteins. It is known that plant PCD does not depend on CASPs because the plant genomes lack CASP homologues. Recent studies have identified several unique plant proteins, *e.g.*, phytaspases from Arabidopsis/tobacco and saspases from oats that are structurally related to subtilisin-like serine proteases, but have CASP-like activities. These proteins are located in extracellular spaces and are able to relocate into cytoplasm in response to cell death signals to execute PCD (Vartapetian et al., 2011). It is not unreasonable to hypothesize that PR-1 proteins

may also contribute to PCD in similar ways if they do function as substrate-specific proteases like CASPs. It would be also interesting to explore the possibility that certain PR-1 proteins may mediate PCD by activating or inactivating other PCD-related extracellular enzymes such as phytaspase/saspase-like proteins. To test these hypotheses further, we have expanded our mutational analysis to other residues conserved among PR-1 and CASPs including the potential catalytic cysteine residue (C113), which is predicted to form a disulfide bridge in P14a (Fernandez et al., 1997) but may be flexible because it is not strictly conserved in the PR-1 domain (data not shown). Microarray-based protease profiling is underway to determine if any of the recombinant PR-1 proteins has CASP-like or other protease activities.

In summary, three function-related new features (dimerization, protease resistance and the CASP-related motif-like sequences) have been identified in the PR-1 proteins of hexaploid bread wheat, an economically important cereal crop prone to devastating pathogen attacks. These new features prompt us to examine the function of plant PR-1 proteins with respect to a potential role in protease-mediated PCD pathways that are of vital importance in diverse host-pathogen interactions.

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